Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6909–6913, August 1993
Chemistry

"Diversomers": An approach to nonpeptide, nonoligomeric chemical diversity

(multiple synthesis/solid-phase synthesis/hydantoin/benzodiazepine)

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Communicated by Pedro Cuatrecasas, April 8, 1993

ABSTRACT **Solid-phase chemistry, organic synthesis, and an apparatus for multiple, simultaneous synthesis have been combined to generate libraries of organic compounds ("diversomers"). Arrays of compounds were synthesized over two to three steps incorporating chemically diverse building blocks on a polystyrene-based solid support in a multiple, simultaneous manner. The generality of this approach is illustrated by the syntheses of dipeptides, hydantoins, and benzodiazepines.**

Recent advances in robotics, miniaturization, and automation have resulted in the development of rapid, high throughput biological screening assays, which can quickly exhaust available sources of chemical diversity. Driven by these advances in biological testing, several methods of generating chemical diversity, primarily peptide- or nucleotide-based oligomer libraries, have been developed using multiple, simultaneous chemical synthesis (1–4) or molecular biology techniques (5–7).

While undeniably useful in chemical lead discovery, these oligomer libraries are limited. The chemical leads discovered from these libraries still require extensive modifications to produce suitable drug candidates due to the general unsuitability of peptides or oligonucleotides as stable, orally active drugs. Furthermore, the available chemical fragments, or building blocks, are generally limited, even allowing for the use of unnatural enantiomers or artificial amino acids and modified nucleotides. Finally, these oligomeric libraries contain a repetitive backbone linkage, either amides or phosphates, which is contrary to the concept of diversity.

Recently, Simón *et al.* (8) reported the development of oligomeric N-substituted glycines as motifs for the generation of compound libraries. Although these libraries offer several advantages over peptide or oligonucleotide libraries—for example, different structural variations, reduced susceptibility to hydrolysis, and the incorporation of achiral building blocks—they still suffer from a repetitive amide motif. One method for the synthesis of nonpeptide, nonoligomeric compounds was recently reported by Bunin and Ellman (9) for the solid-phase synthesis of 1,4-benzodiazepine derivatives. Their method, however, is limited by requisite introduction of auxiliary functionality (e.g., hydroxy or carboxylic acid) in the target molecule to facilitate attachment to the solid support. Furthermore, no apparatus or method for multiple, simultaneous synthesis is described.

True chemical diversity is nly achievable by removing the restrictions on the bond-forming reactions and building blocks used. The resulting compound libraries would have the same advantages and uses as oligomer-based libraries with the added benefit that the target structures are limited only by the chemists' creativity.

We have developed an apparatus and method for the multiple, simultaneous synthesis of organic compounds to create unique collections of compounds, which we call "diversomers." This apparatus and method greatly increases the flexibility and diversity of structures that can be produced by multiple, simultaneous synthesis technology. The target compounds are simultaneously, but separately, synthesized on a solid support in an array format (Fig. 1) to generate an ensemble of structurally related compounds. Using this diversomer method, we have simultaneously synthesized and characterized 40 discrete hydantoins and 40 discrete benzodiazepines. The benzodiazepines were then screened in a competitive binding assay to ascertain biological activity.

MATERIALS AND METHODS

Apparatus. An apparatus suitable for multiple, simultaneous synthesis on a solid support is represented in Fig. 2. The key feature of the apparatus is the use of a gas dispersion tube, which we refer to as a "pin," to physically contain the solid support (resin) during the reactions. This concept of "resin *in* a pin" is contrasted to current solid-phase multiple, simultaneous synthesis technology, which contains the resin in a reservoir well (10, 11) ("resin *in* a well") or on a polypropylene rod (12) ("resin *on* a pin"). Our design overcomes several disadvantages including limited amounts of final products (<<1 mg), the inability to produce soluble products, difficulties in manipulating solid supports, and, in particular, the general unsuitability for normal organic synthesis techniques. The fritted glass filters serve to contain the solid support, allow efficient mixing between reactants in the reservoir wells and the resin in the frit, and facilitate separation of the resin-bound intermediates from excess reagents, solvents, and by-products. The holder block serves to secure the pins and provides a means for simultaneously manipulating the array as a single unit. The reservoir block is constructed with an array of reaction wells that accommodate the fritted glass filters, while concurrently retaining a quantity of reactant necessary to perform the required reactions. Separate reaction wells allow individual reactions to be executed and monitored, while maintaining the integrity of filtrates, intermediates, and final products corresponding to each location in the array. The manifold encloses the upper portion of the pins, allowing control over the reaction atmo-

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Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; ISTD, internal standard; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; Boc, *t*-butyoxycarbonyl; SAR, structure–activity relationship; meq, milliequivalent(s).

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Building Block #1	Building Block #2 A	B	C	A	B	C	A	B	C
1	1AX	1BX	1CX	1AY	1BY	1CY	1AZ	1BZ	1CZ
2	2AX	2BX	2CX	2AY	2BY	2CY	2AZ	2BZ	2CZ
3	3AX	3BX	3CX	3AY	3BY	3CY	3AZ	3BZ	3CZ
	X			Y			Z		

Building Block #3

FIG. 1. Two sets of building blocks (1–3 and A–C) undergo a single bond forming reaction to give nine elements (molecules). A third set of building blocks (X–Z) and a second reaction will provide 27 elements.

sphere. Extended pins can serve as condensers when a chilled gas is circulated through the manifold, thus providing a means to maintain reflux. The apparatus is secured with gaskets and clips to create a gas-tight unit. Reagents can be added directly into the pins by injection through a gasket-sealed plate at the top of the manifold. Due to the heterogeneous nature of these reactions, agitation is critical to success and can be achieved by rotational platform shaking, magnetic stirring, or preferably by sonication.

Automation. A Tecan model 5032 robotic sample processor (Tecan U.S., Research Triangle Park, NC) was used to perform all of the liquid sample handling and TLC spotting.

General Synthesis. 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acid *p*-alkoxybenzyl alcohol resins [Wang resins (13), 200–400 mesh, 1% crosslinked with divinylbenzene, 0.37–0.60 milliequivalent (meq)/g], hydroxymethyl resin (200–400 mesh, 1% crosslinked with divinylbenzene, 1.04 meq/g), and *t*-butoxycarbonyl (Boc)-protected amino acid Merrifield resins (200–400 mesh, 1% crosslinked with divinylbenzene, 0.57–0.89 meq/g) were obtained from Bachem. Other chemicals and solvents were obtained from Aldrich and EM Science and were used without further purification. All synthetic compounds and intermediates gave satisfactory ^{1}H NMR and MS. Gas chromatography was performed on a Varian model 3400 instrument.

The standard-array synthesis cycle begins with weighing an appropriate amount of solid support into each pin and fitting the pins into the holder block equipped with gaskets. The manifold and the reaction wells are attached, and a positive flow of nitrogen is maintained throughout the total reaction sequence. The resins are swelled in the first reaction solvent and are drained by gravity in preparation for the first reaction cycle. Reagents are dispensed, as appropriate, into clean reaction wells or directly into the pins through the gasket-sealed plate at the top of the manifold. The apparatus is heated, cooled, and agitated as necessary to complete or equilibrate the reactions. Reaction times are determined from preliminary validation studies and/or monitoring of filtrates during the course of the reaction [GC or HPLC in conjunction with internal standard (ISTD) calibration methods]. At the end of each reaction the pins are subjected to a series of wash cycles to remove residual solvents, reagents, and by-products. A standard wash protocol includes sequentially submerging the pins in 2 × 3–5 ml each of *N*,*N*-dimethylformamide (DMF), methanol, water, water/dioxane (1:1), dioxane, and CH_2Cl_2, followed by agitation by sonication for 10–15 min, and finally draining by gravity and nitrogen purge. The efficiency of the wash cycles is monitored by TLC or GC/ISTD of the wash filtrates.

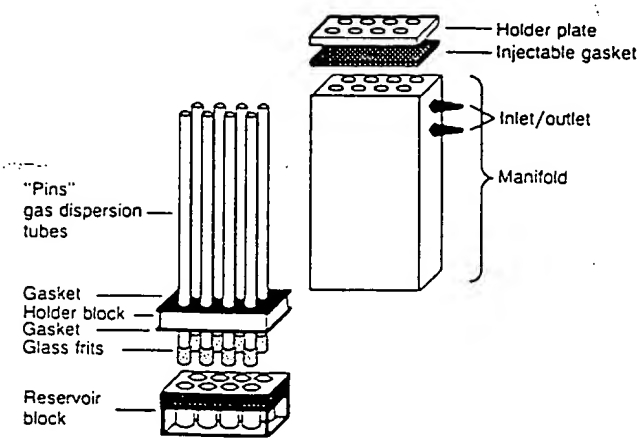


FIG. 2. The apparatus consists of an array of gas dispersion tubes (pins), a reservoir block with multiple reaction wells, a holder block, a manifold, and gaskets.

Synthesis of Hydantoins. Forty discrete hydantoins were synthesized by deprotecting, then treating each of eight amino acid resins with each of five isocyanates. This three-step synthesis creates an array with eight variations in step two and five variations in step three to generate a 40-unit (8 × 5) two-dimensional array (Fig. 3, data not shown).

Approximately 95–105 mg each of seven Fmoc-protected amino acid Wang (13) resins (phenylalanine, glycine, isoleucine, leucine, alanine, valine, and tryptophan) and 95–105 mg of Boc-protected diphenylglycine (prepared by condensation with hydroxymethyl resin) was measured into 40 pins. The resins were swollen with 3 ml of DMF.

To deprotect the Fmoc-amino acids, the appropriate pins were submerged in reaction wells containing 3 ml each of 25% (vol/vol) piperidine/DMF with an ISTD (anthracene). To deprotect the Boc-amino acids, the appropriate pins were submerged in reaction wells containing 3 ml each of 50% (vol/vol) trifluoroacetic acid (TFA)/DMF. The apparatus was agitated for 6 hr in a sonic bath, and the reaction progress was monitored by analyzing aliquots of the filtrates for the Fmoc–piperidine adduct and dibenzofulvene by GC/ISTD calibration methods.

Resin-bound ureas were synthesized by submerging the appropriate pins in reaction wells containing 3 ml each of the appropriate isocyanate in DMF (5–20 eq), with an ISTD (anthracene). The apparatus was agitated in a sonic bath for 6 hr, and the reaction was monitored by derivatization of a sample of the filtrate with an appropriate amine or alcohol, followed by analysis by GC/ISTD calibration methods.

To cyclize and cleave the final hydantoins from the solid support, the pins were submerged in reaction wells containing 3 ml each of 6 M aqueous HCl. The apparatus was heated in an oil bath at 85–100°C for 2 hr, while maintaining a positive chilled nitrogen flow through the manifold.

To isolate and purify the products, the pins were submerged in reaction wells, each containing 3 ml each of methanol. The apparatus was agitated in a sonic bath for 10–15 min to extract the hydantoins from the resins. The methanol extraction protocol was repeated three times, until the filtrates were free of any organic components, as determined by TLC. The HCl and methanol filtrates were concentrated on a Speed-Vac instrument to afford 39 of the 40 desired hydantoins as the HCl salts. A total of 0.3–11.5 mg corresponding to 4–81% yield of the desired hydantoins was isolated and analyzed by TLC, MS, and ^{1}H NMR.

Synthesis of Benzodiazepines. Forty discrete benzodiazepines were synthesized by treating each of five amino acid resins with each of eight 2-amino benzophenone imines. This two-step synthesis created an array with five variations in step one and eight variations in step two to generate a 40-unit (8 × 5) two-dimensional array (Fig. 4 and Table 1).

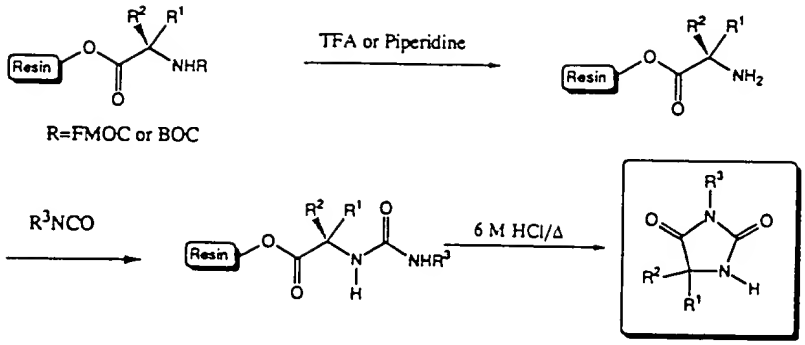


FIG. 3. General route for synthesis of hydantoins.

Five Boc-protected amino acid Merrifield resins (alanine, glycine, phenylalanine, tryptophan, and valine) were deprotected in bulk (1–5 g), using TFA/CH_2Cl_2, 1:1, at 25°C overnight. The resins were washed with dioxane and CH_2Cl_2, then dried under vacuum, and used directly as the TFA salts.

Approximately 99–107 mg each of five-amino acid Merrifield resins prepared above was measured into 40 pins. The resins were swollen with 3 ml each of CH_2Cl_2.

To form the resin-bound imines, the pins were submerged in reaction wells containing 3 ml each of the appropriate 2-aminobenzophenone imines (3–6 eq) in dichloroethane and heated at 60°C (oil-bath temperature) for 24 hr. The pin array was then drained and washed by repeatedly dispensing 4-ml portions of CH_2Cl_2 through the aperture at the top of each pin 12 times, until the washes were no longer colored.

To cyclize and cleave the benzodiazepines from the solid support, the pins were submerged in reaction wells containing 3 ml each of 100% TFA. The apparatus was submerged in an oil bath (60°C) and heated for 20 hr.

To isolate and purify the final products, the pins were drained and then extracted by repeatedly dispensing 2-ml portions of CH_2Cl_2 through the aperture at the top of each pin three times. The combined washes and reaction filtrates were evaporated under a stream of nitrogen. A simple two-phase extraction procedure was implemented using the Tecan 5032 processor. The residues from evaporation were dissolved in 3 ml of CH_2Cl_2 and mixed with 3 ml of saturated aqueous $NaHCO_3$. The organic phase was withdrawn, and the aqueous layer was extracted twice more with 1.5 ml of CH_2Cl_2. The combined organic extracts were dried with $MgSO_4$, filtered, and concentrated as before to yield the expected benzodiazepines. The 40 products were characterized by TLC, ^1H NMR, and MS. A total of 2–14 mg corresponding to 9–63% yield of each benzodiazepine was isolated with estimated purities (by ^1H NMR) typically >90%.

Assay for Inhibition of Fluoronitrazepam. The assay performed by NovaScreen (Scios-Nova Pharmaceutical, Baltimore) employed bovine cortical membranes with [^3H]fluoronitrazepam as the radioligand. The percent inhibition of radioligand binding was determined at three concentrations (10^{-9}, 10^{-7}, and 10^{-5} M). IC_{50} data were calculated for each compound from the average of two determinations at each concentration.

RESULTS AND DISCUSSION

Central to the demonstration of the diversomer library is the development of a "general" method for the multiple, simultaneous synthesis of organic molecules. This method should satisfy the following criteria. The compounds should be simultaneously, but separately, synthesized in an array format (Fig. 1) in a soluble form and in sufficient quantity (>1 mg) and purity to allow multiple *in vitro* biological testing. The apparatus should be compatible with all the normal techniques of organic synthesis. To the extent possible, the method should use automation for speed, accuracy, and precision. Finally, the intermediate products should be readily separable from by-products and excess reagents. To satisfy this last requirement we chose to implement solid-phase synthesis techniques using functionalized, cross-linked polystyrene resins. The overall feasibility of this approach was suggested by literature reports for the synthesis of nonpeptide molecules on resins (14–16).

In the diversomer method, building blocks are sequentially coupled to the growing molecule on the resin until the penultimate, resin-bound product at each location in the array is complete. Cleavage from the resin yields a final product that can be readily separated from the spent resin. Several options for cleavage are illustrated in Fig. 5. The preferred strategy (cleavage 1) constructs a resin-bound penultimate product possessing distal functionality which, when activated or unmasked, will attack the resin-linking bond and eject the cyclized product into solution. Because compounds that do not "react" remain attached to the resin, this option should provide purer final products.

Validation Studies. Initially, a diversomer synthesis requires verification of the proposed resin-based synthesis including several representative examples before an array synthesis is performed. Fourier transform-infrared, enhanced with deconvolution techniques (17–19), and ^{13}C gel-phase NMR (20–22) of the resin-bound intermediates provide an excellent means to directly monitor the progress of reactions on a polystyrene support. These techniques enable qualitative assessment of the success of reactions on a solid support but are not amenable for use within the apparatus. (Their use within the apparatus would require the removal of a prohibitive amount of resin from the pins.) However, analysis of the reaction filtrates by GC or HPLC in conjunc-

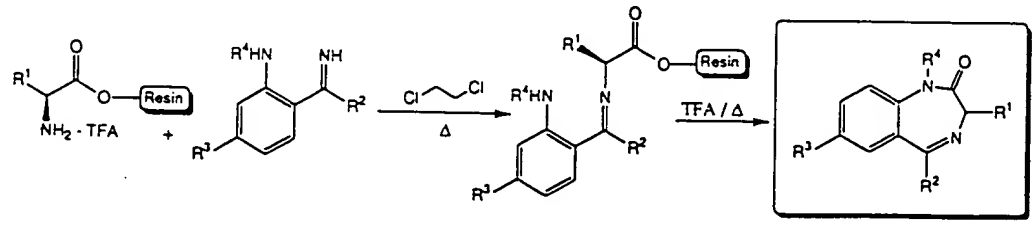
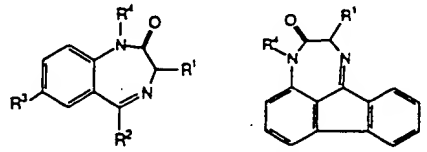


FIG. 4. General route for synthesis of benzodiazepines.

Table 1. Benzodiazepines generated in array



Structure A Structure B

No.	R1*	R2*	R3	R4	Yield†, mg	Yield†, %	IC50‡
1	Me	Ph	H	H	6.1	40	1,700 nM
2	Me	Ph	Cl	H	9.6	56	200 nM
3	Me	4-MeOPh	H	H	5.8	34	69,000 nM
4	Me	Ph	NO_2	H	4.9	28	91 nM
5	Me	B§	B§	H	9.6	63	29,000 nM
6	Me	Ph	Cl	Me	3.2	18	160 nM
7	Me	Chx	H	H	6.4	41	31,000 nM
8	Me	2-Thn	H	H	7.4	47	5,500 nM
9	H	Ph	H	H	9.4	44	1,100 nM
10	H	Ph	Cl	H	13.7	55	19 nM
11	H	4-MeOPh	H	H	5.5	23	33,000 nM
12	H	Ph	NO_2	H	8.0	31	16 nM
13	H	B§	B§	H	3.4	16	44,000 nM
14	H	Ph	Cl	Me	5.2	20	21 nM
15	H	Chx	H	H	7.0	32	6,100 nM
16	H	2-Thn	H	H	8.8	41	940 nM
17	Bzl	Ph	H	H	8.6	52	19,000 nM
18	Bzl	Ph	Cl	H	8.8	46	1,800 nM
19	Bzl	4-MeOPh	H	H	7.3	41	>100 μM
20	Bzl	Ph	NO_2	H	4.9	26	2,400 nM
21	Bzl	B§	B§	H	8.6	52	>100 μM
22	Bzl	Ph	Cl	Me	2.5	13	5,000 nM
23	Bzl	Chx	H	H	6.5	39	>100 μM
24	Bzl	2-Thn	H	H	8.4	48	47,000 nM
25	3-MeInd	Ph	H	H	9.5	43	69,000 nM
26	3-MeInd	Ph	Cl	H	8.0	33	16,000 nM
27	3-MeInd	4-MeOPh	H	H	7.4	31	>100 μM
28	3-MeInd	Ph	NO_2	H	5.8	23	12,000 nM
29	3-MeInd	B§	B§	H	5.2	23	>100 μM
30	3-MeInd	Ph	Cl	Me	2.5	10	14,000 nM
31	3-MeInd	Chx	H	H	7.8	34	>100 μM
32	3-MeInd	2-Thn	H	H	9.2	40	71,000 nM
33	iPr	Ph	H	H	7.1	31	>100 μM
34	iPr	Ph	Cl	H	7.0	28	>100 μM
35	iPr	4-MeOPh	H	H	7.1	29	>100 μM
36	iPr	Ph	NO_2	H	2.2	9	>100 μM
37	iPr	B§	B§	H	6.4	29	>100 μM
38	iPr	Ph	Cl	Me	3.0	11	82,000 nM
39	iPr	Chx	H	H	6.0	27	>100 μM
40	iPr	2-Thn	H	H	8.4	37	>100 μM

*Bzl, benzyl; 3-MeInd, 3-methylindole; 4-MeOPh, 4-methoxyphenyl; Chx, cyclohexyl; 2-Thn, 2-thienyl; iPr, isopropyl.
†Yields are based on indicated loading of commercially available functionalized resins (0.50–0.89 meq/g).
‡Approximate IC_{50} values are based on three-point fit. Values were also obtained for the commercially available diazepam (1.46 nM), nordiazepam (0.2 nM), and nitrazepam (0.67 nM), corresponding to sample numbers 14, 10 and 12, respectively.
§See structure B.

tion with an ISTD provides an indirect method to monitor the course of the individual reactions within the array. This technique provides a means to quantitatively monitor the uptake or release of reagents from the resin.

On the basis of preliminary experiments in the validation stage and consideration of reaction kinetics (e.g., steric or electronic contributions), the reaction conditions are selected to drive the slowest or poorest synthesis in the array to completion.

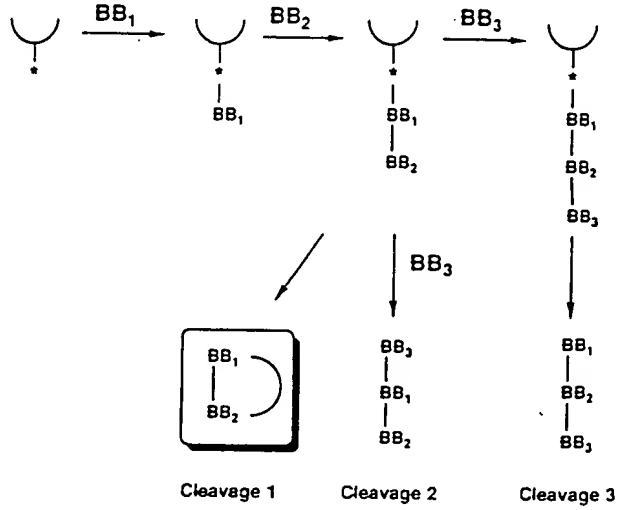


FIG. 5. BB_n represents building blocks that are bifunctional to allow sequential attachment. The curved structure represents the solid support, where the star is the functionality capable of covalently attaching the growing molecule to the solid support. The three possible cleavage modes are illustrated.

The well-established nature of resin-based peptide synthesis (23–25) provided an ideal means to validate the ability of our apparatus to successfully execute multiple, simultaneous reactions. Thus, several arrays of dipeptides were synthesized using standard Fmoc chemistry (26) and a prototype 8-pin apparatus (data not shown). Each product was analyzed by HPLC, MS, and ^1H NMR. The yields (30–85%) and purities were within our defined specifications and, thus, verified the utility of the apparatus to produce the expected and desired products as designed.

Demonstration of Diversomer Method. Although the synthesis of dipeptides serves to demonstrate the utility of the apparatus, it is insufficient to validate the general approach. To validate the diversomer method, we needed to simultaneously synthesize an array of compounds over several synthetic steps, which would produce a representative portion of the known structure–activity relationship (SAR) around a pharmaceutically relevant structure. The syntheses of a series of hydantoins and benzodiazepines were chosen for this demonstration because of the large SAR already developed around the commercial products Dilantin and Valium, respectively.

Synthesis of Hydantoins. The synthesis of an array of 40 hydantoins, including Dilantin, was achieved using the 40-pin apparatus. Five groups of eight Fmoc- or Boc-protected amino acid resins were deprotected and then separately treated with eight groups of five isocyanates, followed by treatment with aqueous 6 M HCl to generate 40 discrete hydantoins (Fig. 3, data not shown).

Some of the key features that demonstrate the strengths, flexibility, and scope of the method and apparatus are as follows. (*i*) The 40 Boc- or Fmoc-protected resin-bound amino acids were simultaneously deprotected. (*ii*) The equilibration or completion of both the Fmoc deprotection and isocyanate reactions was monitored by GC/ISTD calibration methods. (*iii*) The final cleavage and extractions were efficiently performed even in nonswelling solvents. (*iv*) Residual reactants or by-products were removed by wash cycles that included submersion and sonication of the pins in a series of solvents. (*v*) Wash cycles were monitored by robotic spotting of the filtrates on a TLC plate and observing the results under UV light to insure the removal of residual reagents and byproducts.

Synthesis and Testing of Benzodiazepines. The investigation of an array synthesis of benzodiazepines as potential targets was prompted by a brief communication by Camps *et al.* (27), who reported a one-step synthesis of several benzodiazepines starting with a resin-bound amino acid. The synthesis of an array of 40 benzodiazepines related to Valium was achieved by using the 40-pin apparatus. Eight groups of five-amino acid resins were trans-imidated (28, 29) with five groups of eight 2-aminobenzophenone imines (30, 31) to form resin-bound imines, followed by treatment with TFA (32) to generate 40 discrete benzodiazepines (Fig. 4 and Table 1).

To verify that the compounds produced could be used directly in a biological assay, the crude benzodiazepines were assayed, *without* further purification, for inhibition of fluoronitrazepam (Table 1). The compounds expected to exhibit the greatest activity based on the known SAR (33) were the most potent compounds in the array [e.g., R^1 = methyl (1–8) or hydrogen (9–16) and R^3 = chloro (2, 6, 10, 14) or nitro (4, 12)]. The data also provide sufficient semiquantitative information to permit reasonable conclusions concerning the SAR. For example, substituents larger than methyl at R^1 are not tolerated, electron-withdrawing substituents at R^3 are favored, and R^2 appears to be limited to aromatic rings.

Conclusions and Uses. The value of the diversomer approach is represented in the simplicity of the concept, the timeliness for drug discovery efforts, and the innovative combination of methods and apparatus to demonstrate the multiple, simultaneous synthesis of nonpeptide, nonoligomeric compounds on a solid support. The feasibility of preparing arrays of compounds has been demonstrated by the multiple, simultaneous synthesis of 40 discrete hydantoins and 40 discrete benzodiazepines. This result was accomplished with an apparatus capable of performing 40 simultaneous, but separate, chemical reactions on a solid support. The chemistry compatible with the diversomer method encompasses nearly all organic reactions, and the apparatus is sufficiently general and complete to allow for most organic synthesis techniques. The generality and compatibility of this apparatus represents immense improvements over current equipment for multiple, simultaneous synthesis on a solid support (10–12). For example, temperature control, agitation, inert atmosphere, injection of sensitive reagents, and reaction monitoring are an integral part of the apparatus. The key feature of this method is the "resin *in* a pin" apparatus design, which provides a means to simultaneously segregate and manipulate an array of resin-bound intermediates. Furthermore, the simplicity of our apparatus design facilitates the ease of use and construction from commercially available components in a variety of dimensions and multiplicities.

The diversomer method greatly increases the flexibility and diversity of structures that can be produced by multiple, simultaneous synthesis technology. Although the number of compounds produced in a single array (40 separate compounds) is significantly smaller than that which can be prepared as mixtures by some of the current methods for generating peptide libraries (10^5–10^7 peptides), this is adequately offset by the increased quantities and purities of products and the enhanced chemical and structural diversity that can be achieved using the diversomer approach. For example, a typical molecular mass range for orally available drugs is 500–600 g/mol; within this range there are approximately 2.3 million possible tetrapeptides (using 39 D- and L-amino acids), whereas there are an unlimited number of nonpeptide structures. Because each molecule is produced separately and can be fully characterized by standard analytical techniques, no time-consuming deconvolution techniques are needed to determine the active constituent in large mixtures of compounds. Additionally, the quantities produced are sufficient to allow screening in multiple *in vitro* assays.

The diversomer library provides an array of compounds that are directly suitable for biological testing, thereby dramatically enhancing and accelerating SAR development by the ability to screen multiple, related compounds at one time. Well-designed arrays of compounds will provide information necessary for lead compound discovery and SAR evaluation. With continued use, diversomers will provide unlimited sources of chemical diversity.

We are grateful to W. Moos for his leadership in initiation of this research and guidance during its conceptualization, J. Topliss for his long-term advocacy of this type of chemical diversity, and J. Bristol for his continued support. We express our deep gratitude to R. Root-Bernstein for teaching us a new way of looking at research problems and to C. Agree, A. Galen, D. Holsworth, D. Mack, H.-Y. Mei, D. Moreland, and K. Sanders for helpful discussions. We are indebted to R. Harms and D. Van Hofe for their expert craftsmanship in constructing the apparatus.

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